THE ABSORPTION SPECTRA OF SOME BLOODS AND SOLUTIONS CONTAINING HEMOCYANIN

ALFRED C. REDFIELD

(*From the Department of Physiology, Harvard Medical School, Boston and the Marine Biological Laboratory, Woods Hole*)

The present study of the absorption of light by hemocyanin was undertaken in the course of developing a spectrophotometric method for the determination of the quantity of oxygen combined by the blood of invertebrates which contain this pigment. The data obtained provide a precise description of the color characteristic of the body fluids of the various animals examined. Attention has been directed not only to the spectrum of oxygenated blood, which has already been examined with precision in the case of a number of organisms by Dhéré and his collaborators (1919, 1920, 1929), Begemann (1924) and Quagliariello (1922), but also to the apparent absorption of light by reduced blood. The latter observations have led to the conclusion that a very considerable fraction of the light passing through a hemocyanin solution may be scattered by the hemocyanin molecules. The extent of this scattering determines in large part the color characteristic of the various bloods when examined either by reflected or transmitted light. By taking account of the amount of light scattered by the reduced solution, it has been found possible to determine the characteristic absorption spectrum of the molecular complex responsible for the bluish color developed when the hemocyanins combine with oxygen. In this way some information is obtained on the specificity of the oxygen-combining mechanism in the blood of different animals.

Method

Observations have been made upon the blood of the conch, *Busycon canaliculatum*, the horse-shoe crab, *Limulus polyphemus*, the squid, *Loligo pealei*, and the lobster, *Homarus americanus*. The bloods have been drawn by methods previously described (Redfield, Coolidge and Hurd, 1926), and preserved in the cold with toluene until prepared for observation. Under these conditions they may be kept with little change for many days. The bloods have been diluted to concentrations appropriate for the methods involved with sea water, distilled water, or various salt solutions, after which they have been allowed to stand

overnight in the ice box in order to permit equilibrium with the modified environment to be reached. The material has then been filtered and placed in specially constructed tonometers in which it could be brought into equilibrium with various mixtures of gases. Each tonometer consisted of a cylindrical pyrex glass bottle of 200 cc. capacity, to the bottom of which a T-tube was sealed. The ends of the T were ground parallel to one another and were closed with optically flat glass plates sealed in position with DeKhotinsky cement. A chamber was thus provided, having an inside diameter of approximately one centimeter and a length which was in most cases exactly 3.3 centimeters. Following equilibration with the gas mixture, the sample of solution could be run down into the T-tube and the intensity of the light transmitted through it, measured. The specimens were oxygenated by filling the tonometer with oxygen or, in those cases where the character of the oxygen dissociation curve permitted, with air. Solutions containing reduced hemocyanin were prepared by evacuating the bottles after the introduction of the solution and refilling them with hydrogen. The bottles were then rotated for 15 minutes, after which the bottles were re-evacuated and again filled with hydrogen and equilibrated for an additional period of 25 minutes. The precision of the measurements is affected if the solutions are not perfectly clear. For this reason, the greatest care is necessary in filtering the solutions and in being sure that the dissolved materials are in equilibrium with their environment before filtration occurs, as otherwise small amounts of precipitated material may appear in the solutions before the photometric measurements are made. Reduced hemocyanin solutions are particularly troublesome because small amounts of material become insoluble during the mechanical disturbances incidental to evacuation and equilibration of the solutions. Under favorable circumstances, the insoluble particles produced in this way settle out if the specimens are allowed to stand for an hour or more prior to making the measurements. Under other circumstances the solutions remain slightly cloudy and the precision of the measurements is seriously interfered with. The second difficulty is in obtaining complete reduction of the solutions. Reduction appears to be satisfactorily attained by the method outlined above in the case of the bloods. In solutions of purified hemocyanin, because of the change in the shape and position of the oxygen dissociation curve, complete reduction is much more difficult to obtain. Further repetition of the processes of evacuation and equilibration with hydrogen would undoubtedly achieve the desired effect, but unfortunately such repetition increases the amount of insoluble material formed in such solutions and thus defeats its purpose. The use of chemical reducing agents has

not been employed as those which have been tried have led to progressive changes in the color of the reduced material, which again defeats the objects of the experiments.

Measurements of the absorption of light by these solutions have been made with a König-Martens spectrophotometer constructed by Schmidt and Haensch. The light source of the instrument was illuminated by a Mazda projection bulb, the intensity of whose light could be controlled by a rheostat. The width of the slits was kept at 0.2 millimeters except at wave lengths less than 480 mμ, when it was increased to 0.4 or 0.6 millimeters as required in order to secure sufficient illumination. The calibration of the wave length scale of the instrument was checked from time to time and was found at all times to be accurate within 1 mμ. The precision of the instrument was also checked by the determination of the absorption of two colored glass filters, which had been standardized by the U. S. Bureau of Standards.

The absorption of light is indicated by the following equation:

$$\frac{I_0}{I} = \frac{\tan^2 a_0}{\tan^2 a_1}, \tag{1}$$

where I_0 is the intensity of incident light, I the intensity of transmitted light, a_1 the angle of the analyzing prism at which the fields match when a tube containing the solvent is placed in one of the beams of light; a_0 is this angle when the tube containing the solution is placed in this beam. In all cases, a_1 was determined with the absorption vessel filled with distilled water. Test showed that the result was the same, within the limits of observational error, in whichever beam the absorbing solutions were placed. In order to obtain results which might be compared with one another after the blood of different animals was examined, the results have been expressed in terms of the extinction coefficient, E, characteristic of each wave length as defined by

$$\frac{I}{I_0} = 10^{-Ed} \tag{2}$$

where d is the length in centimeters of the column of fluid. It follows that the extinction coefficient, E, is given by:

$$\frac{2(\log \tan a_0 - \log \tan a_1)}{d}. \tag{3}$$

In dealing with the absorption of light by hemocyanin, one is concerned particularly with the absorption of light by the complex formed when oxygen unites with hemocyanin. In this union it has been demonstrated that one atom of oxygen is combined for each atom of copper contained

in the hemocyanin. The union appears to depend upon some grouping in the hemocyanin molecule, of which the copper forms an essential part. For convenience we will refer to this arrangement as the "chromatic group." For purposes of comparison it is interesting to determine the absorption of light in relation to the number of chromatic groups present. According to Beer's Law, the extinction coefficient of a substance in solution is proportional to its concentration. We have consequently expressed the absorption of light by the hemocyanin solutions in terms of E/c, where c is the concentration of copper in the solution expressed as milligram atoms per liter. An advantage of this notation also lies in the fact that the concentration of copper in serum may be readily obtained without the necessity of determining the number of grams of hemocyanin which are present, an investigation which cannot be made unless the hemocyanin of the species has been isolated and properly studied.

The Application of Beer's Law to Hemocyanin Solutions

The foregoing treatment assumes explicitly that in the absorption of light by hemocyanin solutions Beer's Law is valid and that in consequence E/c is a constant characteristic of the substance at each wave

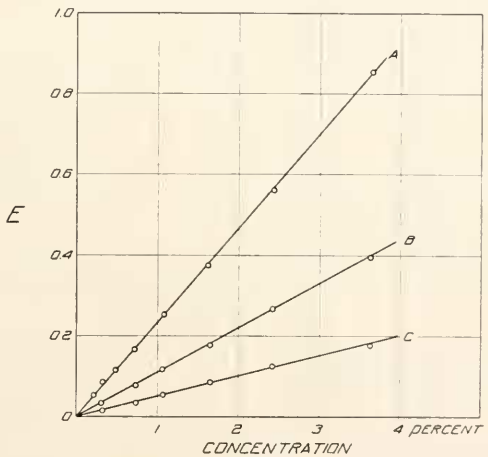

Fig. 1. Extinction coefficient of purified hemocyanin solutions of various concentrations.

A. Busycon canaliculatum at 570 mμ.
B. Limulus polyphemus at 580 mμ.
C. Limulus polyphemus at 480 mμ.

length. Quagliariello (1922) and Svedberg and Heyroth (1929) both present evidence that Beer's Law does not apply in the case of hemocyanin solutions. We have consequently examined this question care-

fully and have found no indication that Beer's Law is not valid when applied to such solutions and to such concentrations and at such wave lengths as we have employed. In Fig. 1 is shown the relation between the extinction coefficient of solutions of purified hemocyanin of two species made at various concentrations. In the case of *Busycon* and of *Limulus* the measurements were made at the wave length of maximal absorption and in the case of *Limulus* also at the wave length at which the absorption is minimal. In all three cases the relation between extinction coefficient and concentration is linear within the accuracy obtainable with photometric measurements on solutions of this

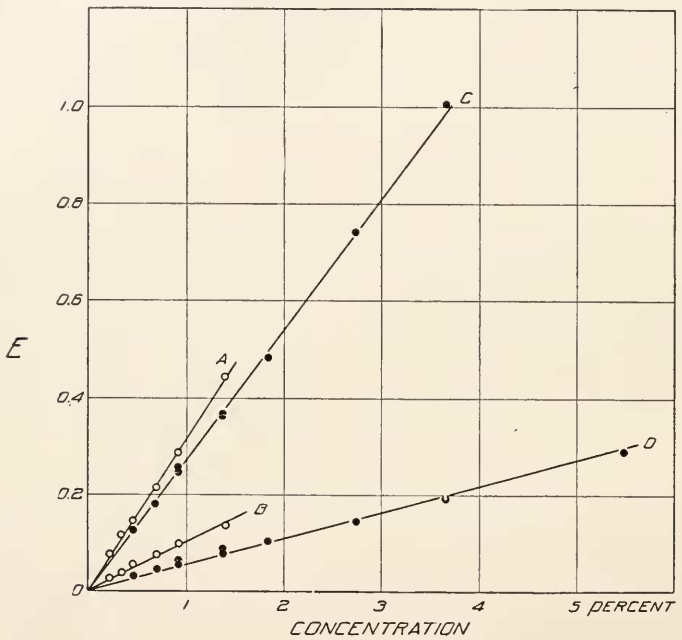

FIG. 2. Extinction coefficient of oxygenated and reduced solutions containing hemocyanin at various concentrations.

A. Busycon canaliculatum serum oxygenated. Dilution with 2.5 per cent NaCl. Wave length 570 mμ.

B. The same, reduced.

C. Busycon canaliculatum hemocyanin in potassium phosphate buffer solution oxygenated. Wave length 570 mμ. Dilution with phosphate buffer, 0.178 molecular phosphate; ionic strength 0.55; molecular fraction as K_2HPO_4 0.90.

D. The same, reduced.

character. Quagliariello's measurements were made upon native blood diluted with 2.5 per cent sodium chloride. It seemed possible that his anomolous results were due to alterations in the environment of the hemocyanin as the result of dilution, which might possibly affect the

degree of scattering of light by the protein, to be subsequently discussed. We have therefore made observations on the serum of *Busycon canaliculatum* similarly diluted with 2.5 per cent sodium chloride and have measured the extinction coefficient not only of the oxygenated but of

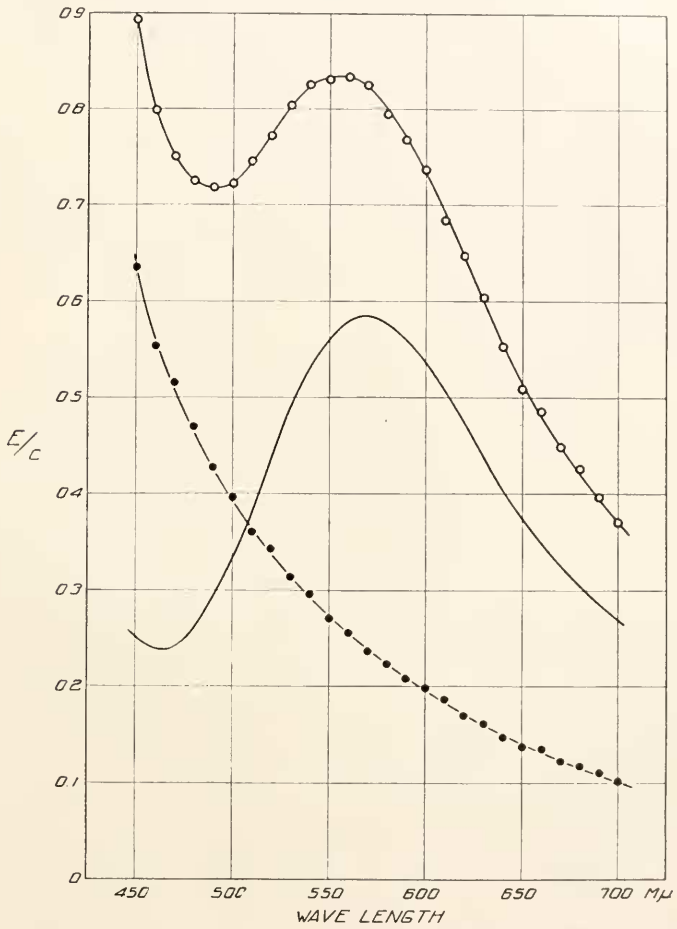

FIG. 3. Absorption spectra of blood of *Busycon canaliculatum*. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, corrected spectrum of chromatic group. Copper content of blood 0.066 mgm. per cc.; dilution, 10 parts blood plus 18 parts H_2O plus 2 parts 0.1N NaOH; pH 9.6; length of absorption vessel 3.3 cm.

the reduced solutions. The results are shown in Fig. 2, curves *A* and *B*. Again it appears that the relation between extinction coefficient and concentration is practically linear. As a further test we have made observations upon a solution of purified *Busycon canaliculatum* hemo-

cyanin dissolved in potassium phosphate buffer and diluted carefully with a similarly buffered solution so as to maintain constant ionic strength. Measurements were made upon both the oxygenated and reduced solutions which again conform closely to the requirements of Beer's Law (Fig. 2, *C* and *D*). We consequently conclude that the assumption of Beer's Law is valid in connection with the observations discussed in this paper.

The Absorption Spectra of Native Blood

The typical spectra of the oxygenated and reduced bloods of *Limulus, Loligo, Busycon* and *Homarus* are presented in Figs. 3, 4, 5 and 6. Detailed descriptions of the solutions will be found in the

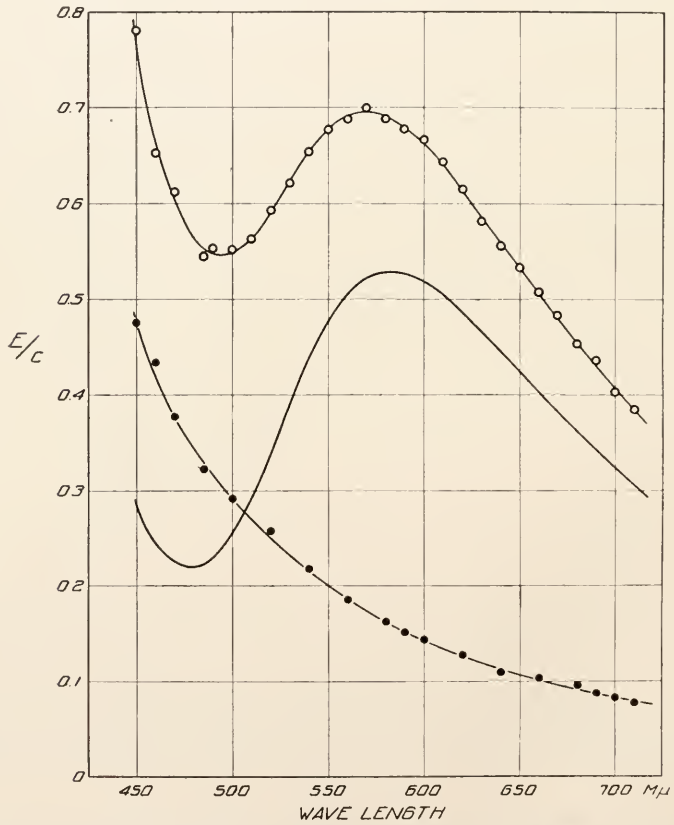

Fig. 4. Absorption spectra of blood of *Limulus polyphemus*. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, corrected spectrum of chromatic group. Copper content of blood 0.081 mgm. per cc.; dilution, 20 parts blood plus 35 parts sea water plus 5 parts 0.08N HCl; pH 6.05; length of absorption vessel 3.3 cm.

legends of these figures. The upper curve in each case represents the absorption of light by the oxygenated blood, the lower curve by the reduced solution. A glance at the curves descriptive of the oxygenated blood serves to show a very considerable difference in the shape of each curve and in the general magnitude of the absorption. The curves do not differ markedly from those described by Quagliariello and others

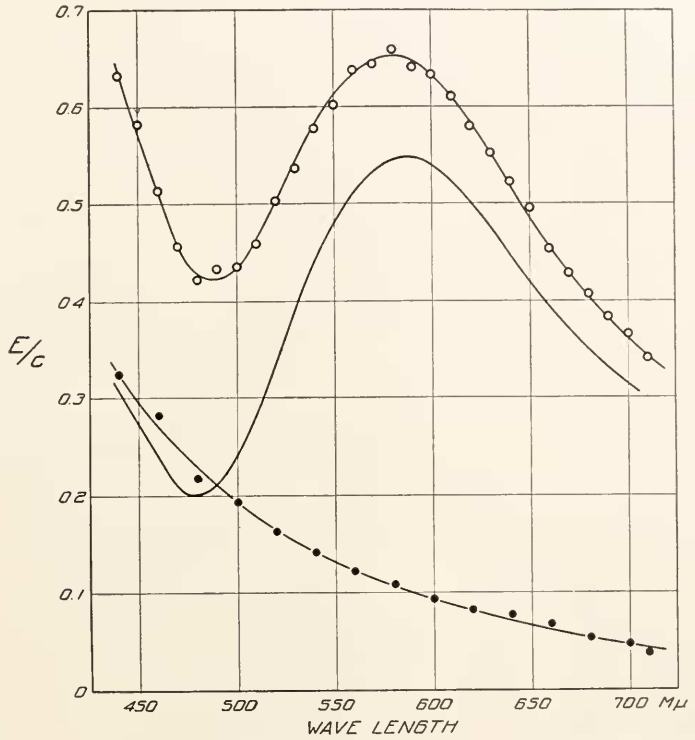

FIG. 5. Absorption spectrum of blood of *Loligo pealei*. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, corrected spectrum of chromatic group. Copper content of blood 0.249 mgm. per cc.; dilution, one part blood plus 6 parts sea water; pH 8.11; length of absorption vessel 3.3 cm.

in the case of European forms belonging to related groups. The curves are alike in displaying a broad band of maximal absorption in the yellow with more or less increased transmission in the region of blue-green. It is in the relative values of the absorption in the blue-green and in the yellow regions that the curves differ characteristically, the species falling in the order *Busycon, Limulus, Loligo, Homarus* as the absorption in the blue-green region decreases. It is, of course, this difference which determines the observed colors of the different bloods.

Spectra of Reduced Bloods

The spectra of the reduced bloods described by the lower curves in Figs. 3, 4, 5 and 6 deserve particular attention. It may be noted in each case, except that of the lobster, that these curves are similar in sweeping with gradual ascent uninterrupted by any obvious absorption bands as one passes from longer to shorter wave lengths. Comparing these curves for the different species, it may be noted that the absorption of light by the reduced blood is greatest in those forms in which the absorption by the oxygenated solution at the blue end of the spectrum is relatively high, the order being again *Busycon, Limulus, Loligo.* This fact may also be related to the observation of Redfield, Coolidge and Hurd (1926) that the Tyndall effect of the bloods studied

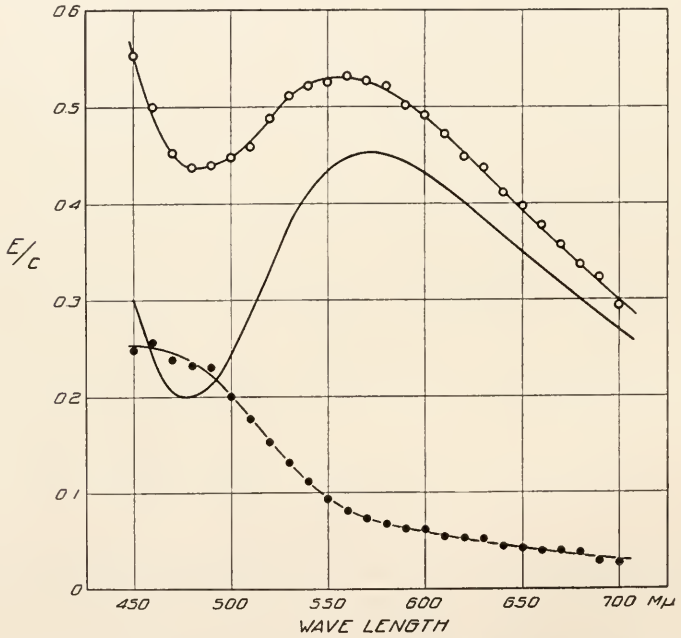

Fig. 6. Absorption spectrum of blood of *Homarus americanus* containing natural pigments. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, corrected spectrum of chromatic group. Copper content of blood 0.0505 mgm. per cc.; dilution, 2 parts of blood plus one part of solution containing 0.4 mols NaCl, 0.01 mols KCl, 0.02 mols $CaCl_2$ per liter; pH 7.87; length of absorption vessel 3.3 cm.

by them decreases in the order *Busycon, Limulus, Loligo* and suggests that the absorption of light by reduced bloods may be due almost entirely to the scattering of light by the solution. The absence of

definite absorption bands in the reduced blood of these three species supports this hypothesis.

According to Lord Rayleigh (Strutt, 1871), when a beam of light passes through a medium containing particles small when compared with the wave length, the light of various wave lengths is scattered in proportion to the reciprocal of the fourth power of the wave length. The light, which is scattered at an angle of 90° from the incident beam, may be expected to be completely polarized provided the particles are spherical. Observation of the Tyndall beam emitted by hemocyanin solutions shows indeed that the Tyndall light is polarized, and inasmuch

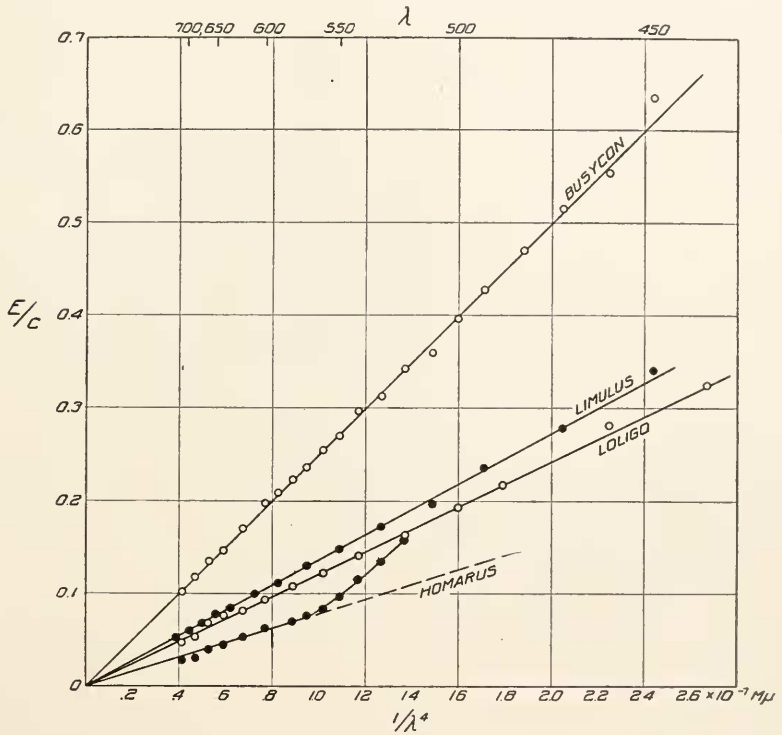

FIG. 7. Extinction coefficients, E/c, of reduced blood plotted against the reciprocal of the fourth power of the wave length, $1/\lambda^4$. For data regarding *Busycon*, *Loligo* and *Homarus* see legends to Figs. 3, 5 and 6. The data for *Limulus* is presented under Fig. 11 at pH 8.77. Concentrations, c, are expressed as milligram atoms of copper per liter.

as the beam disappears entirely when viewed through a properly oriented Nicol prism, the polarization must be very nearly complete. Rayleigh deduces that the attenuation undergone by the beam as the result of

scattering can be expressed by the equation

$$I = I_0 e^{-K\lambda^{-4}x}, \tag{4}$$

where x is the thickness of the scattering medium, λ is the wave length, and K is a constant characteristic of the solution in question. The validity of this equation was demonstrated in the case of mastic solutions by Abney and Festing (1886). Mecklenburg (1915) has shown that solutions of colloidal sulfur scatter light in proportion to the reciprocal of the fourth power of the wave length when the diameter of the particles falls between 5 and 93 mμ. For larger particles the relation no longer holds. The radius of the molecules of hemocyanin of *Helix* and *Limulus,* according to Svedberg and Heyroth (1929), are of the order of 10^{-6} centimeters or 10 mμ, so that we may expect the Rayleigh equation to apply in their case. From inspection of equations 2 and 4, it is obvious that for any given solution E or E/c should be proportional to $1/\lambda^4$. We may consequently test the hypothesis that the apparent absorption of light by bloods containing reduced hemocyanin is due to the scattering of light by the hemocyanin molecules by determining whether E/c at each wave length is proportional to the reciprocal of the fourth power of the wave length. In Fig. 7 the values of E/c for the various reduced bloods are plotted against $1/\lambda^4$. The lines so formed in the case of *Busycon, Limulus* and *Loligo* are straight lines which on exterpolation converge toward and meet at the origin, indicating that the Rayleigh formula does in effect describe the phenomena observed. It may be concluded consequently that the apparent absorption of light by the reduced blood of *Busycon, Limulus* and *Loligo* is to be attributed to the scattering of light by the dissolved hemocyanin.

The Corrected Spectra of the Chromatic Groups

The absorption of light by oxygenated blood must now be attributed to at least two components: the apparent absorption due to scattering and the true absorption due to the chromatic group. If these are the only factors involved, and if it be assumed that the scattering of light by the hemocyanin molecule is unaltered by the process of oxygenation, it is possible to correct the absorption spectra of the oxygenated bloods for the apparent absorption due to scattering and obtain a corrected spectrum of the chromatic group itself. If the attenuation undergone by the beam of light as the result of scattering is given by

$$\frac{I_1}{I_0} = 10^{-E_r d},$$

where I_1 is the intensity of "unscattered" light which would emerge

were no other factors involved, and E_r is the extinction coefficient characteristic of the reduced material; and the further attenuation due to absorption by the chromatic groups is indicated by

$$\frac{I_2}{I_1} = 10^{-E_x d}$$

where I_2 is the final intensity of the emerged beam and E_x is the extinction coefficient expressing the effect of the chromatic group, then

$$\frac{I_2}{I_0} = 10^{-(E_x + E_r)d}.$$

The total absorption of light, however, is given by

$$\frac{I_2}{I_0} = 10^{-E_0 d}$$

where E_0 is the extinction coefficient of the oxygenated solution. Consequently,

$$E_0 = E_x + E_r.$$

The extinction coefficient of the chromatic group at unit concentration is consequently obtained by subtracting the value of E/c for the reduced solution from the value of E/c for the oxygenated solution at each wave length. This has been done, and the results are indicated by the intermediate curves in Figs. 3, 4, 5 and 6.

The Spectra of Blood Containing Other Pigments

The blood of the lobster requires special consideration because in addition to hemocyanin, this blood, in common with that of other crustaceans, contains the pigment tetronerythrin described by Halliburton (1885). Consequently the reduced blood of this species usually has a pinkish color and the bluish hue of the oxygenated blood has a more neutral color than that of the other forms if the pigment is present in sufficient amounts. As the result of the presence of this pigment, the spectrum of reduced lobster blood does not conform to the Rayleigh equation, as the lower curve in Fig. 7 shows. The tetronerythrin may be extracted from the blood by shaking the blood with chloroform. In Fig. 8 the absorption spectrum of the pigment extracted with chloroform is illustrated, the absorption of the dissolved pigment being compared with the absorption when the vessel is filled with chloroform. This substance possesses a maximal absorption at a wave length of 490 mμ and transmits nearly all of the incident light at wave lengths greater than 600 mμ. The apparent absorption of light due to scattering by the reduced blood of the lobster may consequently be arrived at

approximately. By considering the absorption spectrum of the reduced blood at wave lengths greater than 600 mμ, it may be observed from Fig. 7 that these points fall along a straight line drawn from the origin of the diagram. Extending this line beyond 600 mμ indicates the degree of apparent absorption due to scattering at these wave lengths.

The presence of tetronerythrin or similar pigments, the color of which is unaffected by the oxygenation of the blood, does not interfere

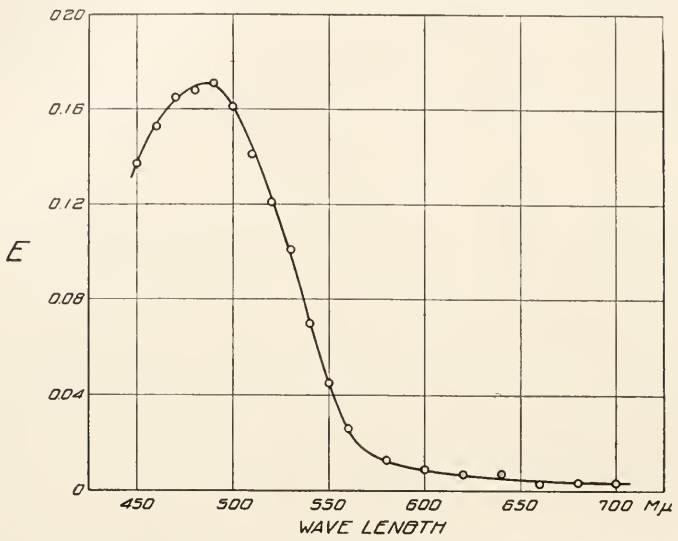

Fig. 8. Absorption spectrum of solution of the pigment extracted from lobster blood with chloroform. Concentration unknown; length of absorption vessel 3.3 cm.

with the determination of the corrected spectrum of the chromatic group. This may be demonstrated by examining the spectrum of blood from which the tetronerythrin has been extracted by chloroform. The spectra of oxygenated and reduced lobster blood so treated are illustrated in Fig. 9. It may be observed that the spectrum of the reduced solution no longer shows the irregularity due to the pigment. The corrected spectrum of the chromatic group may be seen to be almost identical with that obtained from the normal serum illustrated in Fig. 6.

A Comparison of the Spectra of the Chromatic Groups of Different Hemocyanins

It is a question of considerable interest to what extent the various respiratory proteins may be regarded as distinct "inventions of Nature," especially in that it is desirable to know whether the possession

of similar or identical respiratory pigments indicates a generic relation between the groups of organisms possessing them. Recently much evidence has accumulated establishing the fact that the various hemocyanins are specifically different substances. This evidence consists in the demonstration of distinctive differences in the physical and chemical properties of these proteins. On the other hand, the evidence regarding the ratio between oxygen-combining power and copper content of the hemocyanins indicates that these substances have certain points in common, at least with regard to the portion of the molecule concerned with

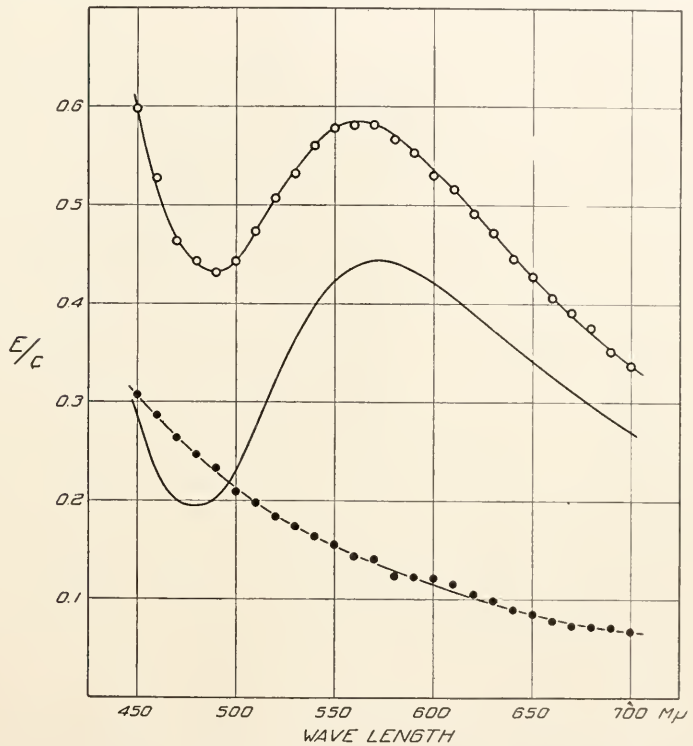

FIG. 9. Absorption spectrum of blood of *Homarus americanus* after extracting the pigment with chloroform. Upper curve, oxygenated blood; lower curve, reduced blood; intermediate curve, the spectrum of the chromatic group. Copper content of blood 0.0522 mgm. per cc.; dilution, 2 parts of blood plus one part of solution containing 0.4 mols NaCl, 0.01 mols KCl, 0.02 mols $CaCl_2$ per liter; pH 8.05; length of absorption vessel 3.3 cm.

this function. To this complex when combined with oxygen we have applied the designation "chromatic group." A comparison of the spectra of the chromatic groups of different forms should consequently give evidence regarding the similarity of the chromatic groups in the

hemocyanins of different classes of animals. In Fig. 10 the corrected spectra of the chromatic groups of the four species which we have studied are collected. It may be seen that on the whole the curves are strikingly alike, not only with regard to their shape, but also in relation to the actual quantity of light absorbed by equal numbers of chromatic

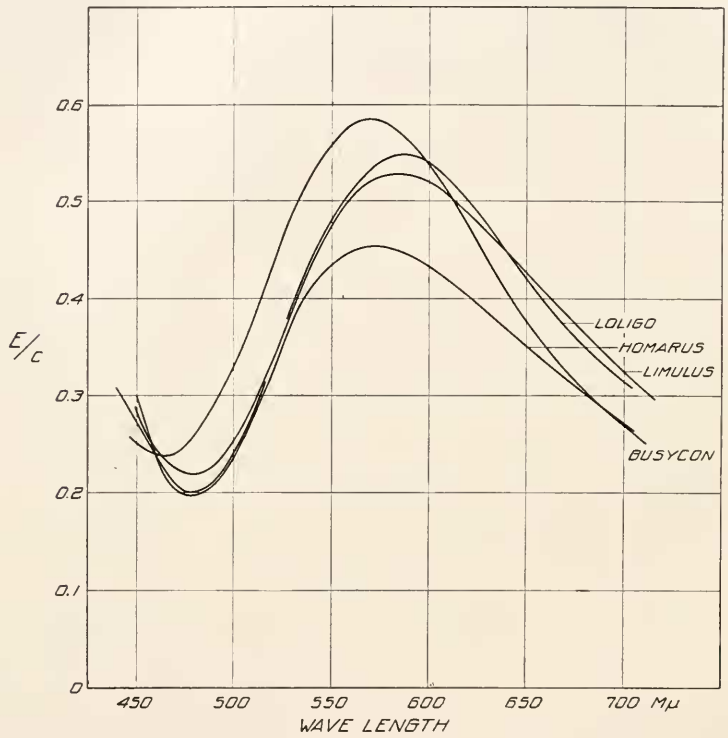

FIG. 10. Absorption spectra of chromatic groups of blood of *Busycon, Limulus, Loligo* and *Homarus*. For data see Figs. 3, 4, 5 and 6.

groups. One is forced to the conclusion that the complexes responsible for these spectra are very much alike in each case. On the other hand, there are unquestionable differences between the spectra in the different cases.

FACTORS AFFECTING THE ABSORPTION OF LIGHT BY THE CHROMATIC GROUP

A comparison of the chromatic groups of different species raises the question as to whether the differences observed may be attributed to differences in the chemical make-up of the body fluids in question.

It is consequently desirable to examine the effect of the nature of the solvent upon the absorption of light by hemocyanin solutions.

Hydrogen Ion Concentration.—The first point to be considered is the influence of hydrogen ion concentration upon absorption and scattering. When specimens of *Limulus* blood, to which various amounts

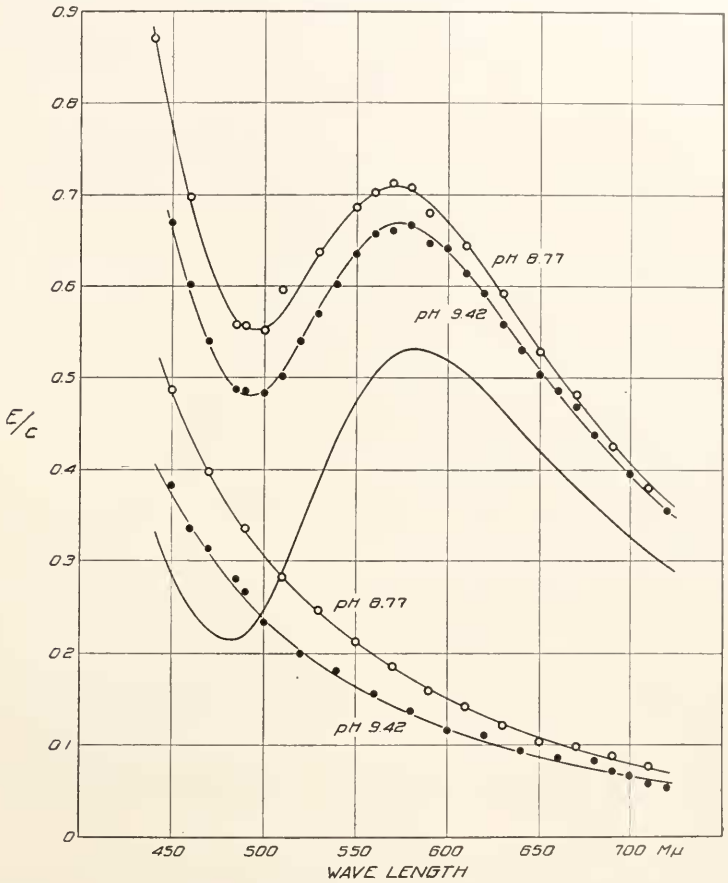

FIG. 11. Absorption spectra of blood of *Limulus polyphemus* at different hydrogen ion concentrations. Upper curves, oxygenated blood at pH 8.77 (hollow circles) and pH 9.42 (dots); lower curves, the same after reduction; intermediate curve, the spectrum of chromatic group, which is identical in both cases. Copper content 0.081 mgm. per cc.; length of absorption vessel 3.3 cm. Dilution which gave pH 8.77: 20 parts blood, 35 parts sea water, 5 parts 0.04N NaOH; dilution which gave pH 9.42: 20 parts blood, 35 parts sea water, 5 parts 0.1N NaOH.

of acid or alkali have been added, are examined, it is obvious to the eye that the color of the solution more alkaline than about pH 9 is different from the others. This difference is evident not only in the

oxygenated, but also in the reduced solutions, the oxygenated solution being a purer blue beyond pH 9 and the reduced solution having a fainter yellow color. In Fig. 11 are illustrated absorption spectra of specimens of oxygenated and reduced *Limulus* blood which were diluted with sea water, to which small quantities of sodium hydroxide had been added so that the solutions were at pH 8.77 and 9.44 respectively. With these curves the data presented in Fig. 4 should be compared, as the latter was obtained from the same blood brought to pH 6.05

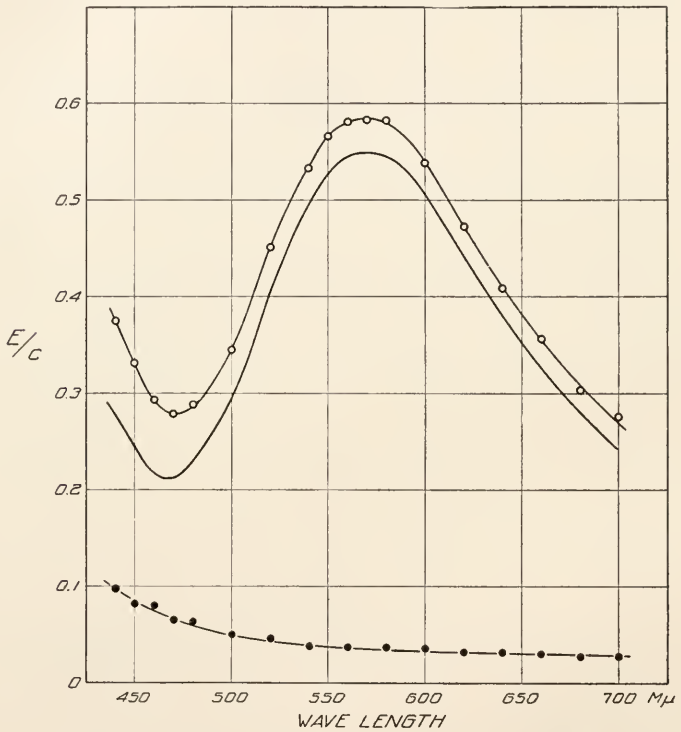

FIG. 12. Spectra of hemocyanin of *Busycon canaliculatum*. Upper curve, oxygenated; lower curve, reduced; intermediate curve, spectrum of the chromatic group. Hemocyanin purified by precipitating four times with saturated ammonium sulfate followed by dialysis. It contained 0.129 grams hemocyanin per cc. and 0.308 mgm. Cu per cc. Dilution, 2 parts hemocyanin solution plus 14 parts H_2O plus one part 0.1N NaOH; pH 9.16; length of absorption vessel 3.3 cm.

by the addition of sea water containing small quantities of hydrochloric acid. The spectra illustrated in Figs. 4 and 11 account for the observed differences in color. The more alkaline specimen absorbs less light than the others in both the oxygenated and the reduced conditions.

It is clear also that the more alkaline solution scatters less light than do the others. Comparison of the corrected spectra of the chromatic groups shows, on the other hand, that the true absorption of light is not changed to a detectable degree by alterations in the hydrogen ion concentration. The differences in the spectra of the oxygenated bloods are sufficiently accounted for by the differences in scattering.

Salts.—A more profound alteration in the solvent may be obtained by purifying the hemocyanin so that it may be dissolved in water

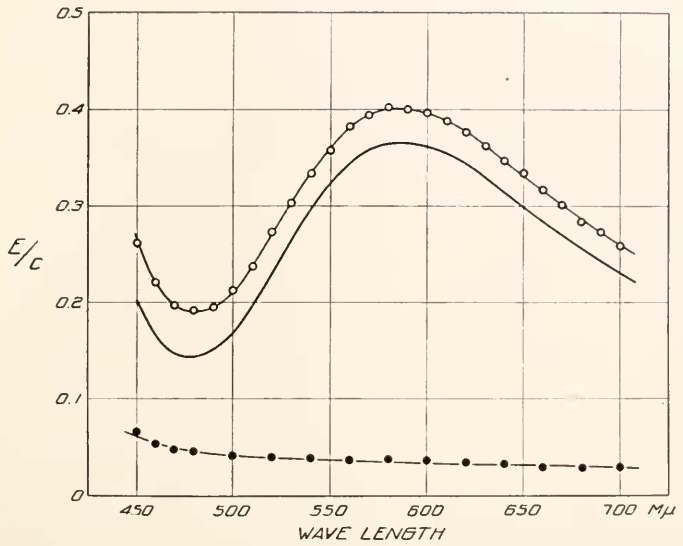

FIG. 13. Spectra of hemocyanin of *Limulus polyphemus*. Upper curve, oxygenated; lower curve, reduced; intermediate curve, spectrum of the chromatic group. Hemocyanin purified by precipitating four times with saturated ammonium sulphate followed by dialysis. It contained 0.109 grams hemocyanin per cc. and 0.184 mgm. Cu per cc. Dilution, 5 parts hemocyanin solution plus 12.5 parts H_2O plus 2.5 parts 0.1N NaOH; pH 9.10; length of absorption vessel 3.3 cm.

practically free of salts or other substances. By this means it is possible to compare the spectra of the chromatic groups of the different hemocyanins in solutions which are more or less identical. When solutions of pure hemocyanin are compared, it may be observed that the Tyndall phenomenon has undergone great diminution. Dilute solutions of reduced hemocyanin are practically colorless. The oxygenated solutions are of a purer blue color than when these substances are dissolved in the blood. These characteristics are all accounted for by an examination of the absorption spectra of the solutions, in which it may be observed that the reduced solutions appear to absorb very little light

and to absorb only slightly more light at the violet end of the spectrum than at the red end. Similarly the transmission of light in the blue-green region of the spectrum of the purified oxygenated hemocyanin is much greater than in the case of blood, and the absorption spectrum of the oxygenated solutions does not differ greatly from those of the corrected spectra of the chromatic groups. Spectra of purified hemocyanin solutions of *Busycon, Limulus* and *Homarus* are illustrated in

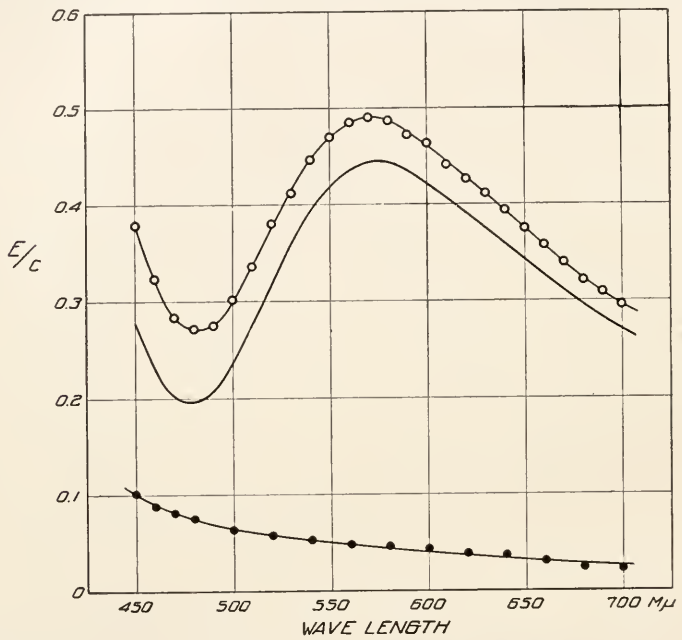

FIG. 14. Spectra of hemocyanin of *Homarus americanus*. Upper curve, oxygenated; lower curve, reduced; intermediate curve, spectrum of the chromatic group. Hemocyanin, purified by dialysis. Solution contained 0.1185 grams dry solids per cc. and 0.196 mgm. Cu per cc. Dilution: one part hemocyanin solution plus 3 parts H_2O; pH 8.10; length of absorption vessel 3.3 cm.

Figs. 12, 13 and 14, together with the corrected spectra of the chromatic groups.

Comparison may now be made between the spectra of the chromatic groups of the purified hemocyanin and of the native blood. This is done in the case of these three species in Tables I, II and III. For accurate comparison the value of E/c for each wave length in the case of the purified hemocyanin is compared with its value in the case of the native blood. If the spectra of the chromatic groups are identical, this ratio should be the same at all wave lengths and have the value 1.0.

Examination of the tables showed that the ratio is not quite constant in each case at different wave lengths. The divergences are not large, but appear to be reproducible and indicate that the spectrum of the chromatic groups undergoes certain small changes as the result of the process of purification. The ratio also deviates from the value of 1.0 in each case. With *Busycon* and *Homarus* the purified material absorbs only slightly less light at each wave length than does a like concentration

TABLE I

Comparison of absorption of light by chromatic groups of blood and purified hemocyanin of Busycon canaliculatum.

Wave Length	Hemocyanin	Blood	Ratio
$m\mu$	E/c	E/c	
460	0.216	0.238	0.908
480	0.230	0.260	0.885
500	0.296	0.333	0.890
520	0.406	0.436	0.932
540	0.497	0.530	0.938
560	0.546	0.580	0.942
580	0.546	0.575	0.950
600	0.506	0.537	0.942
620	0.442	0.473	0.935
640	0.380	0.403	0.942
660	0.325	0.348	0.935
680	0.280	0.304	0.923
700	0.242	0.268	0.904

of hemocyanin in native blood. In the case of *Limulus* the discrepancy is much greater, amounting to about 30 per cent. These differences might be due to an alteration in the absorption of light by each chromatic group. On the other hand, they might be adequately accounted for on the assumption that as the result of the process of purification a certain quantity of the hemocyanin has lost the ability to combine with oxygen, which is necessary in order that the chromatic group be formed. The difference in the case of *Limulus* is sufficiently large to allow this possibility to be tested by a determination of the oxygen-combining power of the solution. The hemocyanin solution employed in this case contained 1.93 milligram atoms of copper per liter and might be expected to have an oxygen capacity of 1.93 milligram atoms of oxygen per liter. Actual analyses of the oxygen content of this solution when equilibrated with air yielded the values, 1.94, 1.95, 1.90 (mean 1.93) milligram atoms of oxygen per liter. Allowing 0.50 milligram atoms of oxygen per liter dissolved in the solution, one obtains 1.43 milligram

atoms as the actual oxygen-combining capacity. This value is 74 per cent of the theoretical, indicating that 26 per cent of the hemocyanin had lost its ability to combine with oxygen. The absorption of light by this solution is approximately 70 per cent of the absorption to be expected from the observations on hemocyanin as it occurs in native blood as Table II shows. It seems clear that in the case of this specimen at least, the discrepancy between the spectrum of blood and of the puri-

TABLE II

Comparison of absorption of light by chromatic groups of hemocyanin and blood of Limulus polyphemus.

Wave Length	Hemocyanin	Blood	Ratio
$m\mu$	E/c	E/c	
460	0.165	0.244	0.677
480	0.144	0.220	0.655
500	0.168	0.257	0.655
520	0.231	0.338	0.683
540	0.298	0.438	0.680
560	0.345	0.506	0.683
580	0.365	0.528	0.692
600	0.362	0.518	0.699
620	0.344	0.486	0.708
640	0.314	0.446	0.705
660	0.284	0.403	0.705
680	0.256	0.362	0.708
700	0.231	0.324	0.714

fied hemocyanin solution is due in large part to the modification of a portion of the hemocyanin in the process of preparation or preservation. The hemocyanin from which this specimen was prepared had been preserved for many months precipitated in half-saturated ammonium sulfate prior to preparation, and unfortunately we have not had an opportunity of re-examining this question with freshly collected hemocyanin.

These results lead to the conclusion that the observed differences in the extinction coefficients of hemocyanin in blood and in purified solutions may be accounted for largely by the denaturation of the hemocyanin in the process of preparation. They do not demonstrate that some difference in the absorption of light by the chromatic groups does not occur. Unfortunately the precision of the available methods for measuring oxygen capacity in these solutions is so low that changes cannot be detected unless they are relatively large. It may be concluded, however, that the spectra of the chromatic groups vary very

little as the result of freeing the solutions from electrolytes and other impurities.

A comparison of the absorption of light by the reduced solutions of purified hemocyanin illustrated in Figs. 12, 13 and 14, with the curves for the absorption of light by the reduced serum of the corresponding species, shows that in the purified preparations, the scattering of light is much less than in the native blood. In the case of the lobster, the values of E/c characteristic of each wave length are, in the

TABLE III

Comparison of absorption of light by chromatic groups of hemocyanin and blood of Homarus americanus.

Wave Length	Hemocyanin	Blood	Ratio
$m\mu$	E/c	E/c	
460	0.232	0.240	0.968
480	0.196	0.202	0.972
500	0.237	0.244	0.972
520	0.317	0.332	0.956
540	0.393	0.410	0.959
560	0.435	0.447	0.975
580	0.444	0.451	0.984
600	0.421	0.432	0.976
620	0.391	0.402	0.973
640	0.358	0.366	0.979
660	0.327	0.333	0.984
680	0.295	0.300	0.984
700	0.270	0.268	1.007

purified serum, about one-half those characteristic of the reduced blood. In the blood of *Busycon* and *Limulus,* the scattering of light is many times greater than in the purified preparations.

The effect of purification upon the scattering of light may be shown to be due primarily to the removal of electrolytes from the solvent of the hemocyanin. By adding salt to purified hemocyanin solutions, the scattering effect is greatly increased. At the same time, the spectrum of the oxygenated solution approaches more nearly that of native blood. The spectrum of the chromatic group, however, appears to remain unchanged. These facts are illustrated by the data in Table IV, in which the values of E/c for oxygenated and reduced solutions of *Busycon* hemocyanin are compared when it is dissolved in water and when it is dissolved in a solution of potassium phosphate of an ionic strength approximately equal to that of native blood.

It may be concluded from the foregoing that the spectrum of the chromatic group is a relatively constant characteristic of hemocyanin solutions, influenced little if at all by the composition of the solvent provided that this does not interfere with the oxygenation of the material. On the other hand, the apparent absorption of light due to scattering varies greatly with the nature of the solvent and particularly with its salt content and hydrogen ion concentration. These facts are essential to the use of photometric methods in examining these solutions. They demonstrate that the measure of the absorption of light by the

TABLE IV

Absorption of light by hemocyanin of Busycon canaliculatum dissolved in potassium phosphate buffer; phosphate concentration, 0.357 molar; pH, 7.7.

Wave Length	Oxygenated in Phosphate	Reduced in Phosphate	Chromatic Group in Phosphate	Chromatic Group—Salt-Free	Ratio
mμ	*E/c*	*E/c*	*E/c*	*E/c*	
460	0.559	0.345	0.214	0.216	1.009
480	0.514	0.288	0.226	0.230	1.017
500	0.544	0.237	0.307	0.296	0.964
520	0.625	0.208	0.417	0.406	0.974
540	0.691	0.178	0.513	0.497	0.969
560	0.722	0.131	0.567	0.546	0.963
580	0.695	0.132	0.563	0.546	0.971
600	0.632	0.118	0.514	0.506	0.985
620	0.560	0.105	0.455	0.442	0.972
640	0.477	0.092	0.385	0.380	0.987
660	0.415	0.078	0.337	0.325	0.965
680	0.361	0.071	0.290	0.280	0.966
700	0.324	0.069	0.255	0.242	0.950

chromatic group may be a reliable index of the concentration of oxyhemocyanin. They also make it clear that in such measurements every precaution must be taken to control and take account of the degree of absorption due to the scattering of light.

In a preliminary report on the present investigation (Redfield, 1929) it was suggested that the relative size of the particles of hemocyanin could be deduced from the scattering of light with the aid of the Rayleigh theory. However, Raman (1927) has developed a theory of scattering by colloidal solutions, in accordance with which it appears possible to relate the observed optical phenomena to the osmotic pressure of the solutions. The experiments of Loeb on gelatin indicate that the variations in osmotic pressure of protein solutions induced by altering the nature of the solvent, which he accounted for by the

considerations involved in Donnan membrane equilibria, are in the necessary direction and have sufficient magnitude to account for the observed variations of scattering in terms of Raman's theory. Until this possibility is examined critically, it is improper to draw inferences

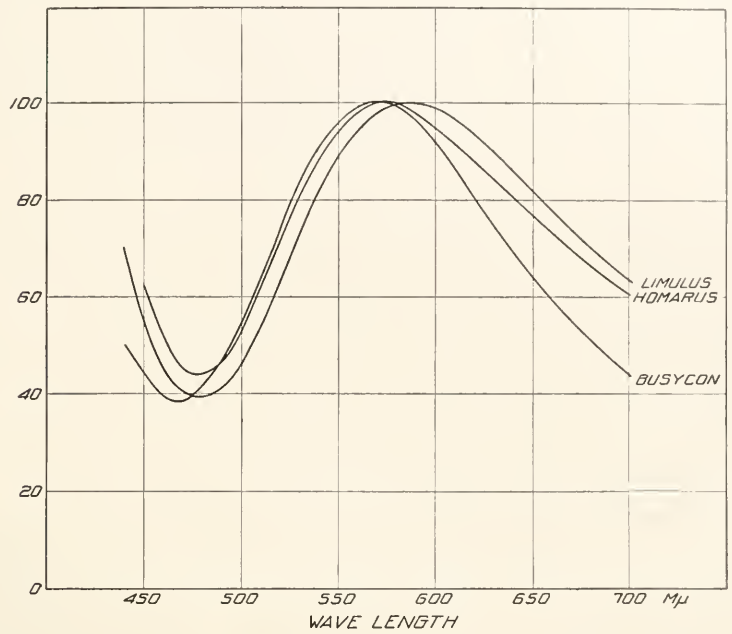

FIG. 15. Absorption spectra of chromatic groups of purified hemocyanins of *Busycon, Limulus,* and *Homarus.* The ordinate is an arbitrary scale such that the value of E/c for each spectrum is 100 at the wave length of maximal absorption. For data see Figs. 12, 13 and 14.

concerning the degree of aggregation of hemocyanin in blood from the phenomenon of scattering.

COMPARISON OF THE CHROMATIC GROUPS OF PURIFIED HEMOCYANIN IN AQUEOUS SOLUTIONS

In order to compare the spectra of the chromatic groups of the different purified hemocyanins it is necessary to employ some method which disregards the errors due to the denaturation of a certain portion of the hemocyanin in the process of purification, as the foregoing discussion indicates that data may not give us accurate information with regard to the concentrations of oxygenated hemocyanin in the various preparations. The spectra of the chromatic groups of the different hemocyanins described by Figs. 12, 13 and 14 have consequently been reduced to an arbitrary scale in which the maximal in-

tensity of absorption in the yellow region has been taken as 100. The data so obtained are plotted in Fig. 15. Comparing these curves, it is evident that even in aqueous solutions the spectra of the chromatic groups are markedly different. One may conclude consequently that the characteristics of these spectra are not dependent upon the chemical peculiarities of the body fluids of the different animals but on specific differences in the chromatic groups themselves or on the influence of the specific characteristics of the hemocyanin molecule as a whole upon that portion which is concerned with the transport of oxygen.

Summary

1. The absorption of light by the blood and by purified preparations of the hemocyanin of the conch, *Busycon canaliculatum*, the horse-shoe crab, *Limulus polyphemus*, the squid, *Loligo pealei*, and the lobster, *Homarus americanus*, has been studied. It is shown that the absorption of light by solutions containing oxygenated hemocyanin may be resolved into two components: (a) that due to the true absorption by the chromatic group formed by the union of oxygen with the portion of the molecule containing copper and (b) that due to the scattering of light by the dissolved protein.

2. In the analysis of the spectrum of the blood of the lobster, the absorption of light by the pigment tetronerythrin has been taken into account.

3. The spectrum of the chromatic group of a given species varies very little, if at all, as the result of alterations in the hydrogen ion concentration and salt content of the solution.

4. The spectra of the chromatic groups of the different species display a considerable similarity, indicating a close chemical relationship. There exist, however, definite differences in the spectra of each species which persist after the process of purification and indicate definite specific differences in the various hemocyanins.

5. The scattering of light varies widely among the different species and is responsible in large part for the difference in appearance of the bloods, particularly when viewed by reflected light. The scattering is modified greatly by changes in the composition of the solution, being diminished in the more alkaline solutions and particularly in solutions free from electrolytes.

REFERENCES

Abney and Festing, 1886. *Proc. Roy. Soc.*, London, **40**: 238.

Begemann, H., 1924. Over de ademhalingsfunctie van haemocyanine, thesis, Utrecht; for abstract see Jordan, H., 1925. *Zeitschr. f. vergl. Physiol.*, **2**: 381.

Dhéré, C. and Burdel, A., 1919. *Jour. physiol. et. path. gén.*, **18**: 685.
Dhéré, C., 1920. *Jour. physiol. et path. gén.*, **19**: 1081.
Dhéré, C., Baumeler, C., and Schneider, A., 1929. *Compt. rend. Soc. de biol.*, **101**: 759.
Halliburton, W. D., 1885. *Jour. Physiol.*, **6**: 300.
Mecklenburg, W., 1915. *Zeitschr. f. Chemie und Industrie der Kolloide*, **16**: 97.
Quagliariello, G., 1922. *Pubblicazioni della Stazione Zoologica di Napoli*, **1**: 57.
Raman, C. V., 1927. *Indian Jour. Physics*, **2**: 1.
Redfield, A. C., 1929. *Am. Jour. Physiol.*, **90**: 489.
Redfield, A. C., Coolidge, T., and Hurd, A. L., 1926. *Jour. Biol. Chem.*, **69**: 475.
Strutt, J. W., 1871. *Phil. Mag.*, **41**: 447.
Svedberg, T., and Heyroth, F. F., 1929. *Jour. Am. Chem. Soc.*, **51**: 539.